

APPENDIX B

Titration of Human AT with Oligosaccharides

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Principle

To human AT is added the synthetic heparin in small amounts. By binding of the pentasaccharide domain to the AT molecule the fluorescence changes until the molecule is saturated. From the saturation point and the shape of the binding curve the dissociation constant (K_d) can be determined. One molecule of pentasaccharide binds to one molecule AT resulting in a conformational change of AT, by which an amino-acid (tryptophan) comes to the outside. This process results in a change of fluorescence.

Methods and Materials

Buffer:	50 mM Tris, 100 mM NaCl (pH 7.5) (Tris75)
AT:	Human AT of ~26 μ M and ~75 μ M, concentration to determined precisely by titration with a known pentasaccharide solution.
Penta:	Pentasaccharide SR-90107-A, 10 mg/ml; i.e. 5.714 mM ($M_r = 1750$). This solution was diluted in Tris75 to 103 μ M (18 μ l/ml).
Samples:	Synthetic heparins are diluted to 100 μ M.
Fluorescence:	The change of fluorescence is followed in a time-trace. Excitation wavelength 285 nm; Emission wavelength 345 nm. The fluorometer: SLM Aminco MC200 Monochromator Spectromic Instruments.
Assay:	Cuvettes are prepared containing 1.9 ml Tris75 and 100 μ l AT. The solutions in the cuvettes are magnetically stirred. To the cuvettes are added the samples/penta in steps of 4 μ l and the fluorescence is recorded.

Results

Experiment 1/12/2003

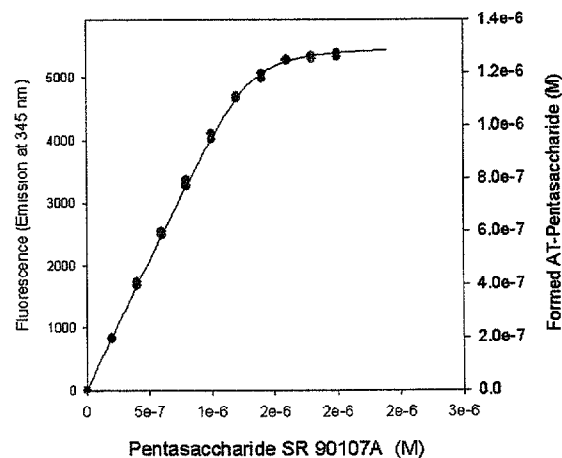
Titration of the AT with pentasaccharide

In Fig. 1 the result of titration AT with pentasaccharide is shown. It is shown that the equivalence point is at 1.308 μ M. This shows that the stock AT is 26.2 μ M. This figure was used for the titration of the synthetic oligosaccharides. The found K_d is 19.0 nM, see Table I.

The drawn line in Fig. 1 is obtained by fitting with equation (i).

Formed AT-penta complex = $\frac{((x+A+K_d) - ((x+A+K_d)^2 - 4 \cdot x \cdot A)^{0.5})/2}{A}$ (i)
in which x is the by fluorescence measured binding of penta, A the AT concentration and K_d the dissociation constant.

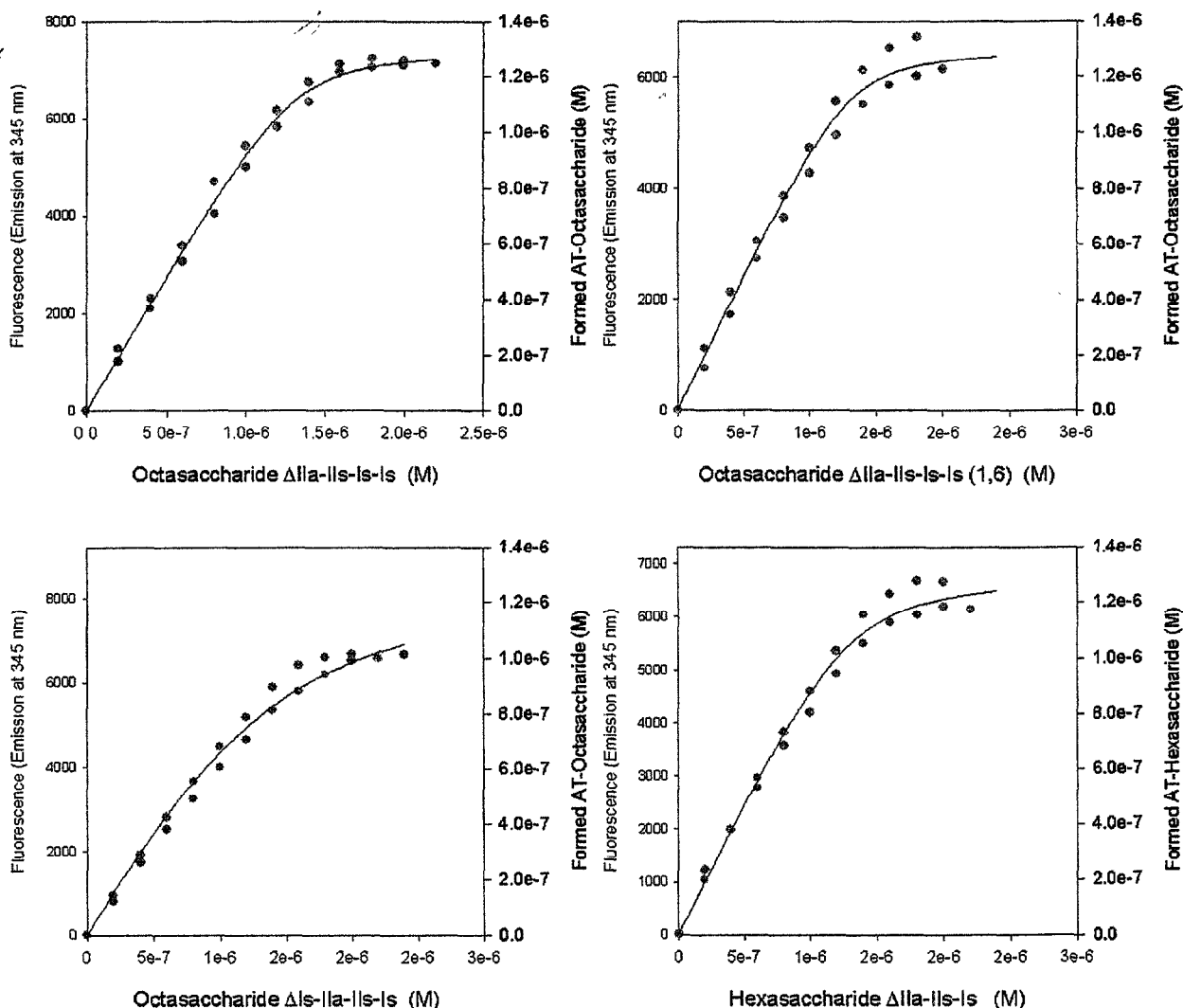
Fig. 1



Titration of AT with oligosaccharides

In Fig. 2 the titrations of AT with the oligosaccharides is shown.

Fig. 2



It is shown that three oligosaccharides have about the same affinity, but somewhat lower than pentasaccharide, for AT and that octasaccharide $\Delta\text{Is-IIa-IIIs-Is}$ has lower affinity for AT. In Table I the results are summarized.

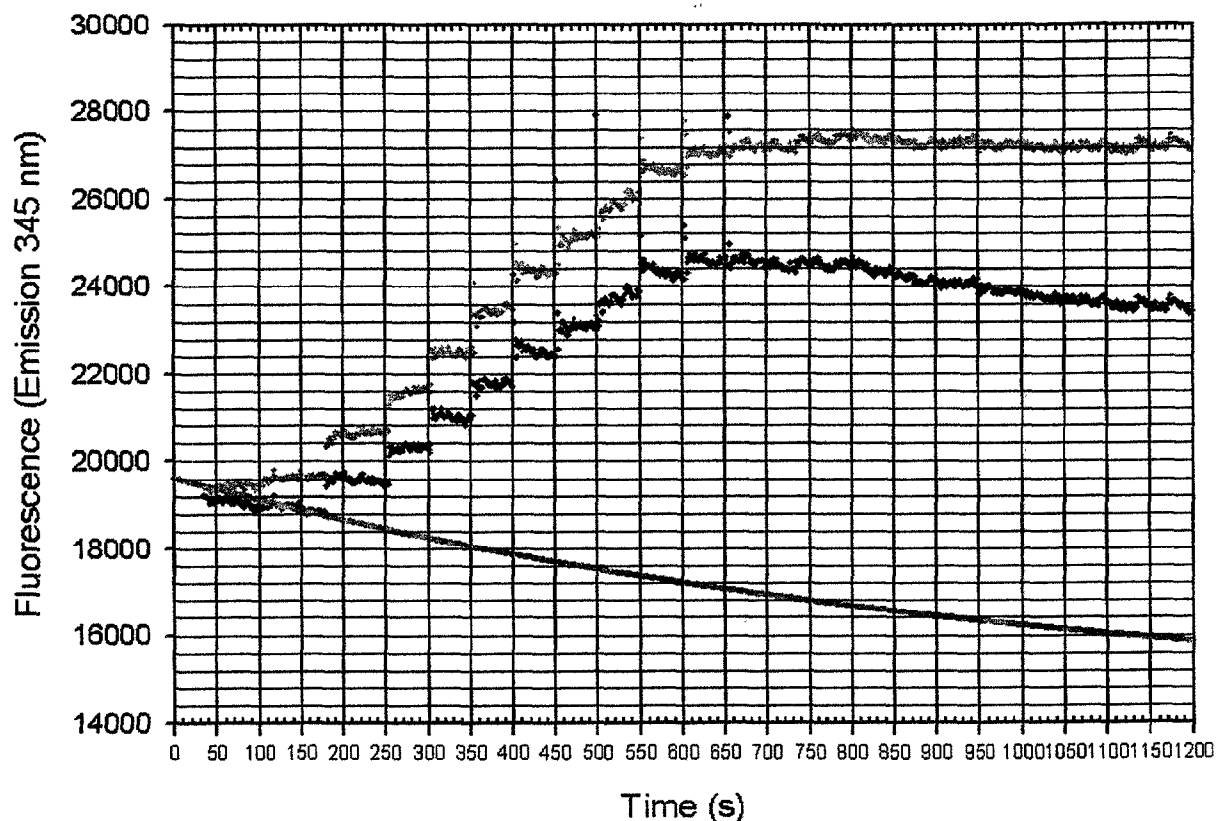
Experiment 15/12/2003

Cuvettes contained: 1960 μl buffer and 40 μl human AT (160202, anti-thrombin activity 50 μM). After 5 min pre-heating at 37 $^{\circ}\text{C}$ the oligosaccharide (100 μM) was added as indicated below. Sequence experiments: 1: Octasaccharide $\Delta\text{IIa-IIIs-Is-Is}$; 2: Octasaccharide $\Delta\text{Is-IIa-IIIs-Is}$; 3: Hexasaccharide $\Delta\text{IIa-IIIs-Is}$; 4: Octasaccharide $\Delta\text{IIa-IIIs-Is-Is (1,6)}$; 5 and 6: SR90107-A; 7: as exp. 4; 8: as exp. 3, 9: as exp. 2, 10: as exp. 1.

The experiment described in Figs. 1-2 were repeated. In this case another batch human AT was used. The concentration was determined as described in Fig. 1: 75.5 μM . The cuvettes now contained 1.51 μM h-AT. To the cuvette was added 4 μl oligosaccharide solution at $t = 200, 250, 300, 350, 400, 450, 500, 550, 600$ and 650 s after starting measuring the fluorescence. Finally 10 μl

solution was added at $t = 750, 850$ and 950 s. During the whole time-trace the solution was stirred and was the $T = 37$ °C. In Fig. 3 an example of a time-trace is shown.

Fig. 3



The black points show the actually measured time-trace. The green points are corrected for quenching. The quenching is assumed to be an exponential decrease and adjusted so much that the last part of the time-trace became horizontal, see also discussion.

In Fig. 4 and Table I the results are shown of the new titration. The result are comparable with the ones described in Figs. 1-2.

On 18/12/2003 the experiments with octasaccharide Δ Is-IIa-IIIs-Is were repeated.

Fig. 4

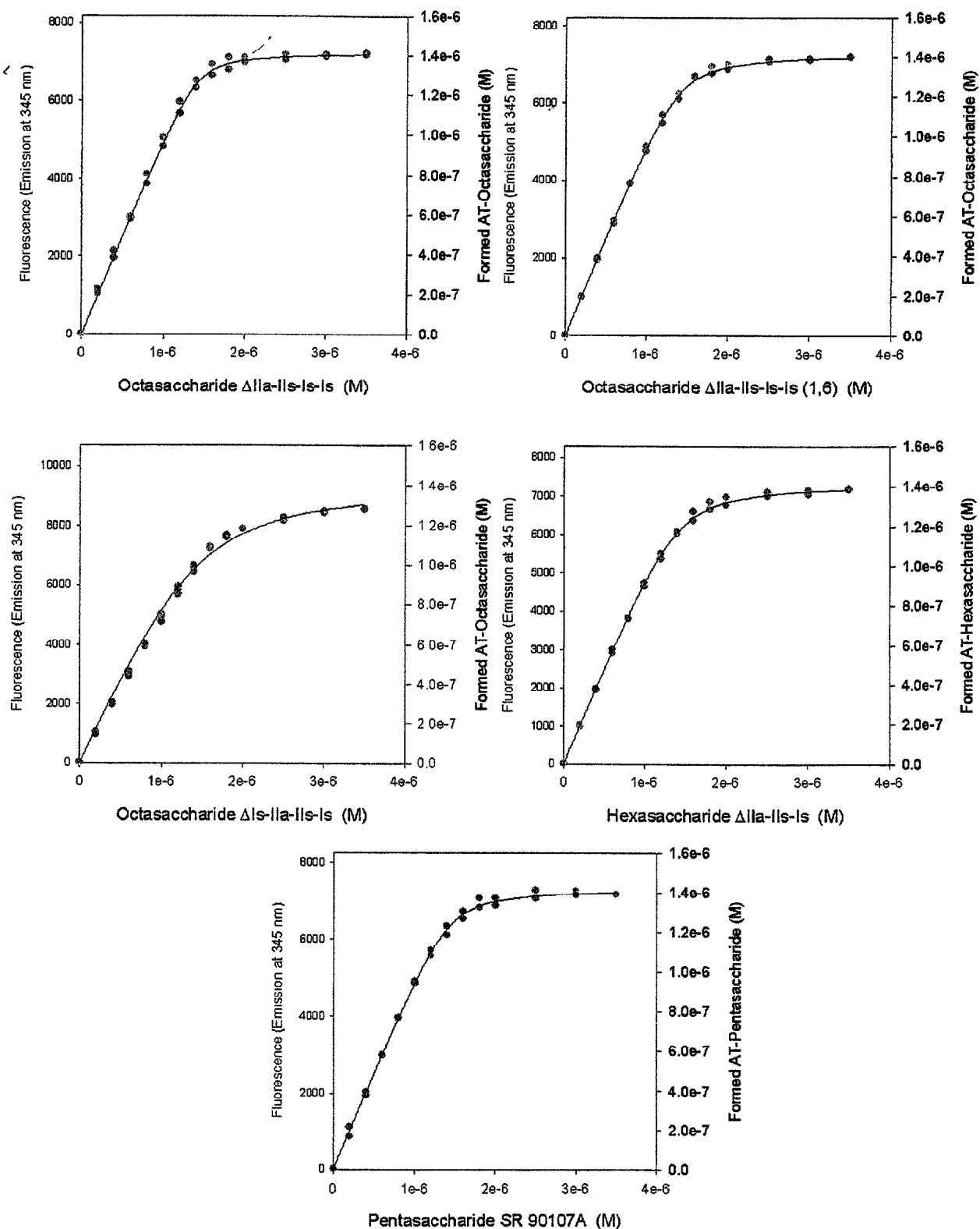


Table I. Affinity of pentasaccharide and synthetic oligosaccharides for human AT. The dissociation constants, the standard error of the K_d's and the binding constants are given. The experiments indicated with (a) give the results of Figs. 1-2 and the ones with (b) the results of Fig. 4.

Compound		Dissociation constant (nM)	(St. Error)	Binding constant (M × 10 ⁻⁶)
Pentasaccharide SR 90107 A	(a)	19	57%	53
	(b)	20	39%	49
Octasaccharide ΔIIa-IIIs-Is-Is (Batch P-32444-019-1)	(a)	34	58%	29
	(b)	13	70%	75
Octasaccharide ΔIIa-IIIs-Is-Is (1,6) (Batch P-32077-080-3)	(a)	32	117%	31
	(b)	18	30%	55
Octasaccharide ΔIs-IIa-IIIs-Is (Batch P-32444-016-1)	(a)	333	82%	3.0
	(b)*	120	18%	8.3
Hexasaccharide ΔIIa-IIIs-Is (Batch P-31404-010-1)	(a)	63	75%	16
	(b)	30	23%	20

*) experiment was done in four-fold

Conclusion

The affinity of pentasaccharide for AT is determined by fitting. In Table I it is shown that the standard error of the dissociation constants is rather high. This is due to the method. The fluorescence of AT alone is about 19000 (under our measuring conditions, but this figure is dependent on the adjustment of the fluorometer) and increases to about 25000 at the saturation point. Moreover, the signal shows quenching during the whole time-trace. We corrected for this effect by assuming that the signal decreases exponentially and adjusted for so much quenching that the last part (from 950 to 1200 s) of the trace became horizontal. In Fig. 3 this is shown.

In Fig. 4 the same set of experiments is shown as in Figs. 1-2. In the latter the results are more reliable, because the titration was continued far above the saturation point. This resulted in lower standard errors in most cases. It is noticed that octasaccharides ΔIIa-IIIs-Is-Is and ΔIIa-IIIs-Is-Is (1,6) have the same affinity for AT as pentasaccharide SR-90107-A. Octasaccharide ΔIs-IIa-IIIs-Is has a 6-16 times lower affinity for AT than pentasaccharide and hexasaccharide ΔIIa-IIIs-Is has a 2-3 times lower affinity for AT than pentasaccharide.

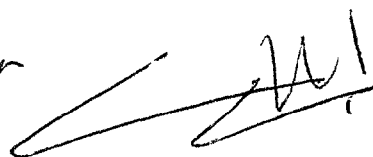
I hereby certify that the experimental studies described and the analyses presented in this report were conducted by me and/or under my supervision.

Name:

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H.C. Hemker



February 9th
2004